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## Engineering of factors determining $\alpha$ -amylase and cyclodextrin glycosyltransferase specificity in the cyclodextrin glycosyltransferase from *Thermoanaerobacterium thermosulfurigenes* EM1

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The starch-degrading enzymes  $\alpha$ -amylase and cyclodextrin glycosyltransferase (CGTase) are functionally and structurally closely related, with CGTases containing two additional domains (called D and E) compared to the three domains of  $\alpha$ -amylases (A, B and C). Amino acid residue 196 (*Thermoanaerobacterium thermosulfurigenes* EM1 CGTase numbering) occupies a dominant position in the active-site cleft. All  $\alpha$ -amylases studied have a small residue at this position (Gly, Leu, Ser, Thr or Val), in contrast to CGTases which have a more bulky aromatic residue (Tyr or Phe) at this position, which is highly conserved. Characterization of the F196G mutant CGTase of *T. thermosulfurigenes* EM1 revealed that, for unknown reasons, apart from the F196G mutation, domain E as well as a part of domain D had become deleted [mutant F196G( $\Delta'$ DE)]. This, nevertheless, did not prevent the purification of a stable and active mutant CGTase protein (62 kDa). The mutant protein was more similar to an  $\alpha$ -amylase protein in terms of the identity of residue 196, and in the domain structure containing, however, some additional C-terminal structure. The mutant showed a strongly reduced temperature optimum. Due to a frameshift mutation in mutant F196G, a separate protein of 19 kDa with the DE domains was also produced. Mutant F196G( $\Delta'$ DE) displayed a strongly reduced raw-starch-binding capacity, similar to the situation in most  $\alpha$ -amylases that lack a raw-starch-binding E domain. Compared to wild-type CGTase, cyclization, coupling and disproportionation activities had become drastically reduced in the mutant F196G( $\Delta'$ DE), but its saccharifying activity had doubled, reaching the highest level ever reported for a CGTase. Under industrial production process conditions, wild-type CGTase converted starch into 35% cyclodextrins and 11% linear oligosaccharides (glucose, maltose and maltotriose), whereas mutant F196G( $\Delta'$ DE) converted starch into 21% cyclodextrins and 18% into linear oligosaccharides. These biochemical characteristics indicate a clear shift from CGTase to  $\alpha$ -amylase specificity.

**Keywords:**  $\alpha$ -amylase; cyclodextrin glycosyltransferase; domain structure; site-directed mutagenesis; product specificity.

Cyclodextrin glycosyltransferase (CGTase) and  $\alpha$ -amylase both belong to glycosyl hydrolase family 13 (the  $\alpha$ -amylase family), which represents a group of ( $\beta/\alpha$ )<sub>8</sub>-barrel proteins (Svensson, 1994). These enzymes are functionally closely related, both catalyzing the degradation of starch by cleavage of  $\alpha$ -1,4-glycosidic bonds. CGTase converts starch mainly into cyclodextrins, cyclic oligomers of 6–8 glucose molecules linked via  $\alpha$ -1,4-glycosidic bonds ( $\alpha$ -,  $\beta$ - and  $\gamma$ -cyclodextrin, respectively). Cyclodextrins have the ability to form inclusion complexes with a wide range of small hydrophobic molecules and may find applications in the food, cosmetic and pharmaceutical industries (Pedersen et al., 1995; Szejtli, 1982). CGTase catalyzes four different reactions, namely cyclization, coupling, disproportionation and hydrolysis (Penninga et al., 1995).  $\alpha$ -amylase converts starch into linear oligosaccharides, resulting in a rapid decrease in viscosity (Antranikian, 1991; Vihinen and Mäntsälä, 1989). The enzyme has found numerous applications in commercial processes, including thinning and liquefaction of starch in the alcohol, brewing and sugar industries.

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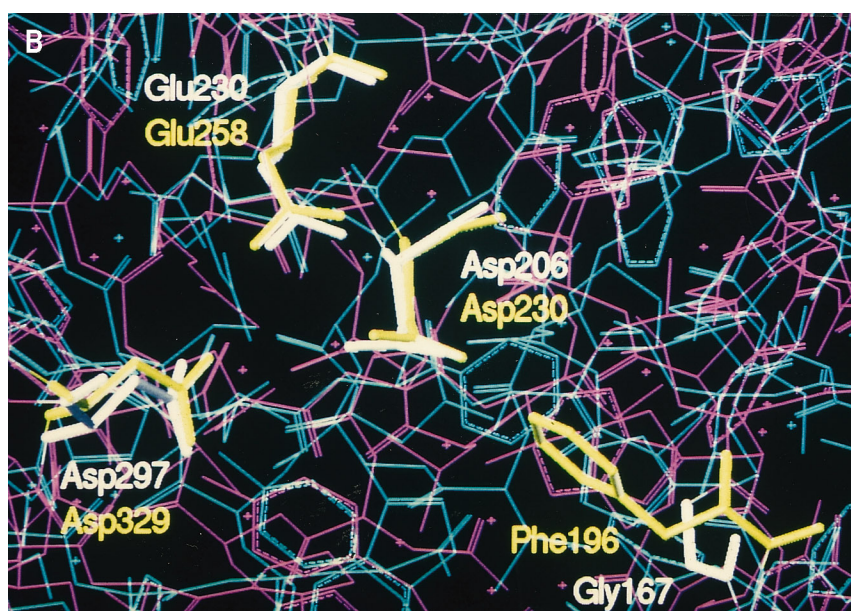
Abbreviations. CGTase, cyclodextrin glycosyltransferase; MBS, maltose-binding site; CD, cyclodextrin.

The crystal structures of several CGTase (Harata et al., 1996; Klein and Schulz, 1991; Knegtel et al., 1996; Kubota et al., 1991; Lawson et al., 1994) and  $\alpha$ -amylase (Machius et al., 1995; Matsuura et al., 1984) proteins have been determined. The primary structures of  $\alpha$ -amylases and CGTases show limited similarity ( $\leq 30\%$ ). In contrast, the three-dimensional structures of the A, B and C domains of CGTases and  $\alpha$ -amylases are quite similar. Compared to  $\alpha$ -amylases, CGTases are much larger and contain two additional domains (D and E). Domain E is involved in raw starch binding (Penninga et al., 1996; Svensson et al., 1989); the precise functions of the D domain remain to be clarified.

Analysis of sequence data has revealed several examples of incorrect classification of CGTases as  $\alpha$ -amylases (Janeček et al., 1995). The  $\alpha$ -amylases from *Bacillus circulans* strain F2 (Fig. 1, Nishizawa et al., 1987) and *Bacillus* sp. strain B1018 were later shown to be CGTases. The  $\alpha$ -amylase from *Thermoanaerobacterium thermosulfurigenes* EM1 has recently been reclassified as a CGTase with an unusually high hydrolytic activity (Wind et al., 1995). The active site amino acids Asp230, Glu258 and Asp329 (*T. thermosulfurigenes* EM1 CGTase numbering), directly involved in catalysis, are fully conserved among the different  $\alpha$ -amylase and CGTase enzymes (Nakamura et al.,

## A

Enzyme	Source	Res	Sequence
$\alpha$ -CGTases	TT	185	SSYEDGIYRNL <b>F</b> ----DLADLNQQ
	BM	185	*TI*****K** <b>Y</b> ----*****I*HN
	KP	177	NDFQVKNH***----**S*****S
	BST	184	**L*****--*****H*
$\alpha/\beta$ -CGTase	BLI	184	*TI*N***K** <b>Y</b> ----*****HN
$\beta$ -CGTases	BC251	184	*TT*N***K** <b>Y</b> ----*****HN
	BC8	184	*SL*N***K** <b>Y</b> ----*****F*HN
	BSP1011	184	*TI*N***K** <b>Y</b> ----*****HN
CGTase	BACCI	185	*TLKN***K** <b>Y</b> ----*****HN
$\alpha$ -Amylase	TAA	154	EDQTQVEDCW*GNTVS**P**DTT
	ANI	156	DNLTMVEDCWEGDTIVS*P**DTT
	AMYBLI	171	IYKFQ*KAYDY <b>L</b> ----MY**IDYD
	AMYPYG	152	NDPYQVRDGCQ <b>V</b> ----L*L**ALE
	AMYHUMANS	155	NDATQVRDCR* <b>S</b> ----G*L**ALG
	AMYHUMANP	152	NDATQVRDCR* <b>T</b> ----G*L**ALE



**Fig. 1. Active-site sequences and catalytic residues.** (A) Alignment of part of the active-site amino acid sequences of several CGTases and  $\alpha$ -amylases (from Penninga et al., 1995 with modifications). \* Indicates an exact match; TT, *T. thermosulfurigenes* EM1; BM, *Bacillus macerans* (Takano et al., 1986); KP, *Klebsiella pneumoniae* (Binder et al., 1986); BST, *B. stearothermophilus* (Kubota et al., 1991); BLI, *B. licheniformis* (Hill et al., 1990); BC251, *B. circulans* strain 251 (Lawson et al., 1994); BC8, *B. circulans* strain 8 (Bender, 1990b); BSP1011, *Bacillus* sp. strain 1011 (Kimura et al., 1987); BACCI, *B. circulans* strain F-2 (Nishizawa et al., 1987); TAA, *Aspergillus oryzae* Taka-amylase A (Nagashima et al., 1992); ANI, *Aspergillus niger* acid  $\alpha$ -amylase (PDB entry 2AAA); AMYBLI, *Bacillus licheniformis*  $\alpha$ -amylase (PDB entry 1VJS); AMYPYG, pig  $\alpha$ -amylase (Nakajima et al., 1986); AMYHUMANS, human saliva  $\alpha$ -amylase (Nakajima et al., 1986); AMYHUMANP, human pancreatic  $\alpha$ -amylase (PDB entry 1HNY). Alignments with ANI, AMYBLI and AMYHUMANP were obtained by 3D structure alignment (Holm and Sander, 1996) with TT (PDB entry 1CIU). (B) Alignment of the catalytic residues of the CGTase from *Thermoanaerobacterium thermosulfurigenes* EM1 (Asp230, Glu258, Asp329; Knegt et al., 1995) and  $\alpha$ -amylase from *Aspergillus niger* (Asp206, Glu230, Asp297; Brady et al., 1991). C $\alpha$  backbone traces are shown. Active-site residues are presented in bold. Blue and yellow: *Thermoanaerobacterium* CGTase; purple and white: *Aspergillus*  $\alpha$ -amylase. Residues Phe196 of the CGTase and Gly167 of the  $\alpha$ -amylase are overlapping.

1992; Strokopytov et al., 1995; Svensson, 1994). It has remained unclear what determines the different product specificities of  $\alpha$ -amylases and CGTases.

Several reports describe the effects of deletions in the C-terminus of CGTase. Deletion of 36, 84, 125 and 225 amino acids from the C-terminus of a *B. circulans* var. *alkalophilus* CGTase yielded inactive proteins (Hellman et al., 1990). Fusions

with *Escherichia coli* alkaline phosphatase, however, increased the specific activity of these truncated proteins again, indicating that the deleted sequences may have a role in maintaining structural integrity. Also, the characteristics of site-directed mutants of the alkalophilic *Bacillus* sp. no. 1011 CGTase, with 10–13 amino acids deleted from the C-terminus, have been reported (Kimura et al., 1989). All mutants produced larger amounts of

glucose, oligosaccharides and  $\alpha$ -cyclodextrin from starch than the parental CGTase, suggesting that the C-terminal domain is important for an efficient cyclization reaction. In contrast, deletion of the C-terminal 90 amino acids from a *Klebsiella pneumoniae* CGTase yielded an active protein not very different from the wild-type enzyme (Bender, 1990a).

Alignment of amino acid sequences from CGTases and  $\alpha$ -amylases suggested that residue 196 (*T. thermosulfurigenes* EM1 CGTase numbering) might play a role in cyclization of oligosaccharides (Penninga et al., 1995). Residue 196 is present at a dominant position in the active-site cleft (Schmidt et al., 1997). All  $\alpha$ -amylases studied have a small residue at this position (Gly, Leu, Ser, Thr or Val; Nakajima et al., 1986), in contrast to CGTases, which have a more bulky aromatic residue (Tyr or Phe) at an equivalent position, which is highly conserved (Penninga et al., 1995). An alignment of part of the active-site amino acid sequences of several CGTases and  $\alpha$ -amylases is given in Fig. 1A. A structural alignment of the catalytic residues of the CGTase from *T. thermosulfurigenes* EM1 and the  $\alpha$ -amylase from *Aspergillus niger* showed that Phe196 of the CGTase is at an equivalent position with Gly167 of the  $\alpha$ -amylase (Wind, 1997; Fig. 1B). Previous studies showed that the presence of an aromatic residue at position 196 is important for an efficient cyclization reaction (Fujiwara et al., 1992; Nakamura et al., 1994; Penninga et al., 1995; Sin et al., 1994). Penninga and co-workers (1995) reported enhanced production of linear oligosaccharides (glucose through maltotetraose) by the site-directed mutants Y196G, Y196W and Y196L of the *B. circulans* strain 251 CGTase.

This study describes construction of a *T. thermosulfurigenes* EM1 mutant CGTase (Phe196Gly) using site-directed mutagenesis. Its characterization revealed that, for unknown reasons, domain E and a part of domain D had become deleted.

## MATERIALS AND METHODS

**Bacterial strains, plasmids and growth conditions.** *E. coli* JM109 (Yanisch-Perron et al., 1985) was used for recombinant DNA manipulations. *E. coli* PC1990 (Lazzaroni and Portalier, 1979), known to leak periplasmic proteins because of a mutation in its *tolB* locus, was used for (extracellular) production of CGTase (mutant) proteins. Plasmid pCT2, a derivative of pUC18 containing the *amyA* (*cgt*) gene of *T. thermosulfurigenes* EM1 (Haeckel and Bahl, 1989; Wind et al., 1995), was used for site-directed mutagenesis, sequencing and expression of wild-type and mutant CGTase proteins. Plasmid-carrying bacterial strains were grown on Luria Bertani medium with 100  $\mu$ g/ml ampicillin. When appropriate, isopropyl- $\beta$ -D-thiogalactopyranoside was added at a concentration of 0.1 mM for induction of protein expression.

**DNA manipulations.** DNA manipulations and transformation of *E. coli* were essentially as described by Sambrook et al. (1989). Electrotransformation of *E. coli* was performed using the Bio-Rad gene pulser apparatus (Bio-Rad). The selected conditions were 2.5 kV, 25  $\mu$ F and 200  $\Omega$ .

**Site-directed mutagenesis.** The mutant CGTase gene (F196G) was constructed via a double PCR method using *Pfu* DNA polymerase (Stratagene). A first PCR reaction was carried out with the mutagenesis primer for the coding strand plus a primer 195–715-bp downstream on the template strand. The reaction product was subsequently used as primer in a second PCR reaction together with a primer 295–815-bp upstream on the coding strand. The product of the last reaction was cut with *NcoI* and *MunI*, and exchanged with the corresponding fragment (900 bp) from the vector pCT2. The resulting (mutant) plasmid

was transformed to *E. coli* JM109 for sequencing and to *E. coli* PC1990 for production of the (mutant) proteins. The following oligonucleotide was used to produce the mutation:

F196G 5'-GCATTTATCGTAACCTAGGTGATTTAGCAG-3'

Successful mutagenesis resulted in appearance of the underlined *AvrII* restriction site, which allowed rapid screening of potential mutants. The mutation was verified by DNA sequencing (Sanger et al., 1977). All 900 bp on the *MunI*–*NcoI* fragment obtained by PCR were checked by DNA sequencing.

**Production and purification of CGTase proteins.** For production of CGTase proteins, *E. coli* PC1990 (pCT2) was grown in a 2-liter fermentor at pH 7.0 and 30°C. The medium contained 2% (by mass) trypton (Oxoid), 1% (by mass) yeast extract (Oxoid), 1% (by mass) sodium chloride, 1% (by mass) casein hydrolysate (Merck), 100  $\mu$ g/l ampicillin and 0.1 mM isopropyl- $\beta$ -D-thiogalactopyranoside. Growth was monitored by measuring the absorbance at 450 nm. At an  $A_{450}$  nm of 2–3, 50 g trypton was added to the fermentor. Cells were harvested after 20–24 h growth (8000 g, 30 min, 4°C), at  $A_{450}$  values of 8–12. The supernatant was directly applied to an  $\alpha$ -cyclodextrin–Sephrose 6FF affinity column (Monma et al., 1988) for further purification of CGTase proteins. After washing the column with 10 mM sodium acetate pH 5.5, the CGTase was eluted with the same buffer supplemented with 1% (by mass)  $\alpha$ -cyclodextrin. The purity and molecular mass of the CGTase (mutant) proteins were checked on SDS/PAGE (Wind et al., 1995). 10  $\mu$ l purified protein was applied to the SDS/polyacrylamide gel containing 3–5  $\mu$ g protein. Protein concentrations were determined by the method of Bradford, using the Coomassie protein assay reagent of Pierce (Pierce Europe bv).

**N-terminal amino acid sequences.** For determination of the N-terminal amino acid sequences, proteins were cut out from SDS/PAGE gels. Elution was performed overnight in 0.1% SDS at 37°C. The N-terminal amino acid sequence was determined at the Gas Phase Sequenator Facility (Department of Medical Biochemistry, University of Leiden, The Netherlands). The instrument used was an Applied Biosystems model 470A protein sequencer, equipped on-line with a model-120A phenothiohydantoin analyzer.

**Enzyme assays.** Specific assays were used to determine the activities of the four different reactions catalyzed by CGTases. In the cyclization reaction, the reducing end of a sugar is transferred to another sugar residue in the same oligosaccharide chain, resulting in the formation of cyclic compounds. Coupling is the reverse reaction in which a cyclodextrin molecule is linked to a linear oligosaccharide chain, producing a longer oligosaccharide chain. In the disproportionation reaction, part of a linear donor oligosaccharide is transferred to a linear acceptor chain. The saccharifying activity is the hydrolysis of starch into linear oligosaccharides.

All assays were standardly performed at pH 5.9 and 60°C. In all cases, initial enzyme activities were measured in the first 5 min of the reaction by taking samples every 1 min, to assure that the rate of the reaction was linear. Cyclization and saccharifying assays were performed as described by Penninga et al. (1995). Coupling activity was measured essentially as described by Nakamura et al. (1993).  $\beta$ -cyclodextrin (2.5 mM) was used as donor substrate and methyl  $\alpha$ -D-glucopyranoside (100 mM) as acceptor substrate. The linear oligosaccharide formed in the reaction was converted to single glucose units by the action of amyloglucosidase (Sigma). Glucose was detected with the glucose/GOD-Perid method of Boehringer Mannheim. Disproportionation activity was measured as described by Nakamura et al. (1994). EPS, 4-nitrophenyl- $\alpha$ -D-maltoheptaoside-4-6-O-ethyl-

**Table 1. Purification of *T. thermosulfurigenes* EM1 wild-type and mutant F196G( $\Delta'$ DE) CGTase proteins.** 2-L supernatant was used for protein purification.  $\beta$ -cyclodextrin-forming specific (sp. act.) and total activities (tot. act.) are shown.

CGTase	Supernatant activity		Purified protein fractions		Purification factor	Yield	Pure protein
	specific	total	specific	total			
	U/mg	U	U/mg	U	-fold	%	mg
Wild-type	0.80	300	165	80	205	25	0.5
F196G( $\Delta'$ DE)	0.15	40	40	20	270	50	0.5

idene (3 mM, Boehringer Mannheim), was used as donor substrate and maltose (10 mM) as acceptor substrate. The reaction product containing the nitrophenyl group was cleaved by the action of  $\alpha$ -glucosidase (Boehringer Mannheim). For each reaction, units were defined as the amount of enzyme producing/converting 1  $\mu$ mol product/substrate at pH 5.9 and 60°C.

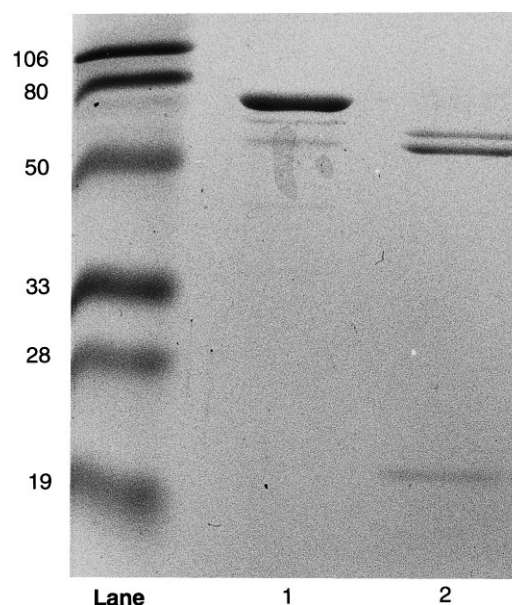
Raw starch-binding properties were studied at standard assay conditions as described by Penninga et al. (1996). An appropriate amount of CGTase was incubated with increasing amounts of granular potato starch (AVEBE) at 4°C for 1 hour (equilibrium was reached within 10 min). CGTase bound to the starch granules was spun down at 4°C for 1 min at 10000 g and the remaining cyclization activity in the supernatant was measured as described.

The pH optimum for cyclization was determined by incubating 0.1 U/ml ( $\beta$ -cyclodextrin-forming activity) of the enzyme with 5% Paselli SA2 (partially hydrolyzed potato starch, AVEBE) in a 10-mM sodium citrate solution set at a specific pH (range 4.0–8.0). For each pH, a new calibration curve was prepared with 0–2 mM  $\beta$ -cyclodextrin. The pH optimum for the saccharifying reaction was determined in a similar way.

**HPLC product analysis.** Formation of cyclodextrins was measured under industrial production process conditions by incubation of 0.1 U/ml CGTase ( $\beta$ -cyclodextrin-forming activity) with 10% Paselli WA4 (pregelatinized drum-dried starch with a high degree of polymerization; AVEBE) in 10 mM sodium citrate, pH 6.0, at 60°C for 45 hours. Samples were taken at regular time intervals and boiled for 10 min. Products formed were analyzed by HPLC, using a 25-cm Econosil-NH<sub>2</sub> 10- $\mu$ m column (Alltech Nederland bv) eluted with acetonitrile/water (65:45, by vol.) at 1 ml/min. Products were detected by a refractive index detector (Waters 410, Waters Chromatography Division). The temperature of the flow cell and column was set at 50°C to avoid possible precipitation of starch. Formation of linear products was directly analyzed. Formation of cyclodextrins was analyzed after incubation of the samples with an appropriate amount of  $\beta$ -amylase (type-IB from Sweet potato, Sigma), degrading linear sugars (but not cyclodextrins) to glucose. The retention times for  $\alpha$ -,  $\beta$ - and  $\gamma$ -cyclodextrins were the same as those for maltotetraose, maltopentaose and maltohexaose, respectively.

## RESULTS AND DISCUSSION

**Construction of mutant F196G.** To study the role of residue 196 in the *T. thermosulfurigenes* EM1 CGTase, Phe196 was replaced by Gly (Table 1). The purity and molecular mass of wild-type CGTase and F196G mutant CGTase were checked on SDS/PAGE (Fig. 2). Wild-type CGTase has a molecular mass of 75 kDa, but displays a molecular mass of 68 kDa on SDS/PAGE (Wind et al., 1995). The minor protein bands were earlier shown to be CGTase degradation products (Fig. 2, lane 1; Wind et al., 1995). To our surprise the mutant F196G preparation displayed

**Fig. 2. SDS/PAGE of purified CGTase (mutant) proteins from *T. thermosulfurigenes* EM1.** Lane 1, wild-type CGTase; lane 2, mutant F196G. Molecular-mass standards are given on the left.

a major protein band with a molecular mass of 54 kDa and a minor protein band of 19 kDa on SDS/PAGE. DNA sequencing of mutant F196G revealed a 460-bp longer gene than that found for wild-type CGTase. For unknown reasons base pairs 1209–1669 of the *cgt* gene had become inserted again behind base pair 1669 in the *cgt* gene, causing a shift in the reading frame and resulting in the stop codon TAA after 11 amino acids (Fig. 3). The expressed F196G protein hence contained 556 amino acids from the N-terminus and a tail of 11 amino acids at the C-terminus (KLLMVLLSNVG; 567 amino acids in total), whereas wild-type CGTase contains 683 amino acids (Fig. 3). The molecular mass of the obtained construct was calculated as 62 kDa, which is in good agreement with the size of the major protein band (54 kDa) found on SDS/PAGE (Fig. 2). The identity of the smaller upper band is unknown (Fig. 2, lane 2). The truncated mutant F196G [F196G( $\Delta'$ DE)] thus lacked all 104 amino acids of domain E and the last 23 amino acids of domain D (out of a total of 84 amino acids), very similar to the situation in  $\alpha$ -amylases in general.

The minor protein (19 kDa) found on SDS/PAGE resulted from a translational restart at Met508 of the *cgt* gene, yielding a protein of 175 amino acids containing the complete E domain and 71 amino acids of domain D. The N-terminal sequence of the 19-kDa protein was determined and confirmed the restart at Met508. Binding of the protein to the  $\alpha$ -cyclodextrin–Sephacrose 6FF affinity column might be explained by the presence of maltose-binding sites (MBS) in the E domain of the CGTase from

	10	20	30	40	50
TBACT	ASDTAVSNVV	NYSTDVIYQI	VTRDFVDGNT	SNNPTGDLVD	PTHSTLKKYF
F196G(Δ'DE)	ASDTAVSNVV	NYSTDVIYQI	VTRDFVDGNT	SNNPTGDLVD	PTHSTLKKYF
	60	70	80	90	100
TBACT	GGDWQGIINK	INDGYLTGMG	VTAIWISQPV	ENIYAVLPDS	TFGGSTSYHG
F196G(Δ'DE)	GGDWQGIINK	INDGYLTGMG	VTAIWISQPV	ENIYAVLPDS	TFGGSTSYHG
	110	120	130	140	150
TBACT	YWARDFKRTN	PYFGSFTDFQ	NLINTAHAHN	IKVIIDFAPN	HTSPASETDP
F196G(Δ'DE)	YWARDFKRTN	PYFGSFTDFQ	NLINTAHAHN	IKVIIDFAPN	HTSPASETDP
	160	170	180	190	200
TBACT	TYAENGRLYD	NGTLLGGVTN	DTNGYFHHYG	GTDFSSYEDG	IYRNLFDLAD
F196G(Δ'DE)	TYAENGRLYD	NGTLLGGVTN	DTNGYFHHYG	GTDFSSYEDG	IYRNLFDLAD
	210	220	230	240	250
TBACT	LNQQNSTIDS	YLKSAIKVWL	DMGIDGIRLD	AVKHMPFGWQ	KNFMDSILSY
F196G(Δ'DE)	LNQQNSTIDS	YLKSAIKVWL	DMGIDGIRLD	AVKHMPFGWQ	KNFMDSILSY
	260	270	280	290	300
TBACT	RPVFTFGWEF	LGTNEIDVNN	TYFANESGMS	LLDFRFSQKV	RQVFRDNTDT
F196G(Δ'DE)	RPVFTFGWEF	LGTNEIDVNN	TYFANESGMS	LLDFRFSQKV	RQVFRDNTDT
	310	320	330	340	350
TBACT	MYGLDSMIQS	TASDYNFIND	MVTFIDNHDM	DRFYNGGSTR	PVEQALAFTL
F196G(Δ'DE)	MYGLDSMIQS	TASDYNFIND	MVTFIDNHDM	DRFYNGGSTR	PVEQALAFTL
	360	370	380	390	400
TBACT	TSRGVPAIYY	GTEQYMTGNG	DPYNRAMMTS	FNTSTTAYNV	IKKLAPLRKS
F196G(Δ'DE)	TSRGVPAIYY	GTEQYMTGNG	DPYNRAMMTS	FNTSTTAYNV	IKKLAPLRKS
	410	420	430	440	450
TBACT	NPAIAYGTTQ	QRWINNDVYI	YERKFGNNVA	LVAINRNLSL	SYNITGLYTA
F196G(Δ'DE)	NPAIAYGTTQ	QRWINNDVYI	YERKFGNNVA	LVAINRNLSL	SYNITGLYTA
	460	470	480	490	500
TBACT	LPAGTYTDLV	GGLLNGNSIS	VASDGSVTPF	TLSSAGEVAVW	QYVSSNSPL
F196G(Δ'DE)	LPAGTYTDLV	GGLLNGNSIS	VASDGSVTPF	TLSSAGEVAVW	QYVSSNSPL
	510	520	530	540	550
TBACT	IGHVGPTMTK	AGQTIIDGR	GFGTSGQVL	FGSTAGTIVS	WDDTEVKVKV
F196G(Δ'DE)	IGHVGPTMTK	AGQTIIDGR	GFGTSGQVL	FGSTAGTIVS	WDDTEVKVKV
	560	570	580	590	600
TBACT	PSVTPGKYNI	SLKTSSGATS	NTYNNINILT	GNQICVRFV	NNASTVYGEN
F196G(Δ'DE)	PSVTPGKILM	VLLSNVG*	Domain D	Domain E	
	610	620	630	640	650
TBACT	VYLTGNVAEL	GNWDTSKAIG	PMFNQVYQY	PTWYDVSVP	AGTTIQFKFI
	660	670	680	683	
TBACT	KKNGNTITWE	GGSNHPTVTP	SSSTGTIVNV	WQQ*	

**Fig. 3. Alignment of the amino acid sequences of wild-type CGTase from *T. thermosulfurigenes* EM1 and mutant F196G(Δ'DE) (without signal peptides).** The start and end of domains A–E and residue 196 are marked.

*T. thermosulfurigenes* EM1. Cyclodextrins were found to bind strongly to MBS1 and MBS2 in the E domain of homologous *B. circulans* strain 251 CGTase (Knegt et al., 1995). Amino acids involved in both binding sites (Trp609 and Trp655 in MBS1, Tyr626 in MBS2) are highly conserved in CGTases (Penninga et al., 1996).

**Characterization of mutant F196G(Δ'DE).** Mutant F196G(Δ'DE) displayed reduced cyclization, coupling and disproportionation activities, compared to the wild-type CGTase. The *T. thermosulfurigenes* EM1 wild-type CGTase possesses an unusually high saccharifying activity, initially resulting in its misidentification as an  $\alpha$ -amylase (Haeckel and Bahl, 1988; Knegt et al., 1996; Wind et al., 1995). Saccharifying activities of CGTases known from literature are much lower, i.e. the wild-type CGTase from *B. circulans* strain 251 displays a saccharifying activity of 3.0 U/mg (Penninga et al., 1995) and the wild-type CGTase from *B. stearrowthermophilus* displays a saccharifying activity of 1.88 U/mg (Fujiwara et al., 1992).

We now observed that, compared to wild-type, the saccharifying activity of mutant F196G(Δ'DE) had doubled (Table 2).

At optimal pH, the mutant enzyme displayed a saccharifying activity of 65 U/mg, the highest ever reported for a CGTase. The mutant enzyme appeared to be relatively stable since activities did not significantly decrease within one month of storage at 4°C. Mutant Y196G of *B. circulans* strain 251 also displayed severely reduced cyclization, coupling and disproportionation activities, compared to the wild-type CGTase (van Alebeek, G. J., unpublished results; Penninga et al., 1995). The saccharifying activities of the *B. circulans* strain 251 wild-type CGTase, however, are relatively minor (3 U/mg), and this activity was enhanced by a factor 1.4 in mutant Y196G and by a factor of 1.6 in mutant Y196L (4.3 U/mg and 4.8 U/mg, respectively; Penninga et al., 1995). Both the F196G mutation and loss of (part of) the D, E domains thus may contribute to the doubling of the saccharifying activity of *T. thermosulfurigenes* EM1 mutant F196G(Δ'DE).

The presence of an aromatic residue at CGTase position 196 thus is crucial for an efficient cyclization reaction. Mutations at position 196 also cause changes in cyclodextrin product ratios. In fact, the size of residue 196 may influence the size of the preferred cyclodextrin formed. Replacement of residue 196 by Leu indeed resulted in production of increased amounts of  $\beta$ -cyclodextrin and  $\gamma$ -cyclodextrin and decreased amounts of  $\alpha$ -cyclodextrin in other CGTases (Nakamura et al., 1994; Penninga et al., 1995; Sin et al., 1994). The cyclodextrin product ratio of the F196G(Δ'DE) mutant enzyme had not significantly changed compared to the wild-type enzyme (Table 3, Fig. 4). Similar observations were made for mutant Y196G of *B. circulans* 251 CGTase (Penninga et al., 1995).

Most CGTases (e.g. the *B. circulans* strain 251 enzyme; Penninga et al., 1995) incubated with starch under industrial process conditions produce only cyclodextrins and no or minor amounts of linear oligosaccharides. The wild-type *T. thermosulfurigenes* EM1 CGTase is quite exceptional, converting starch for 11% into linear sugars (glucose, maltose and maltotriose). This value is even higher for mutant F196G(Δ'DE) (18%; Table 3). Mutant Y196G of the *B. circulans* 251 CGTase also showed a drastically increased conversion of starch into linear saccharides (glucose, maltose, maltotriose and maltotetraose), from 0% for the wild-type enzyme to 16–20% for the mutant enzyme (Penninga et al., 1995).

The pH optimum for hydrolysis has shifted from pH 4.0 to pH 5.0 for wild-type CGTase, and from pH 5.0 to pH 5.5 for mutant F196G(Δ'DE) (Table 2). Also, the pH optimum for cyclization has shifted to a higher pH (from pH 4.5–6.5 to pH 5.5–6.5; Table 2). What is the cause of these shifts in pH? As site-directed mutations at position 196 in the *B. circulans* strain 251 CGTase did not cause structural rearrangements (Penninga et al., 1995), we expect that in the *T. thermosulfurigenes* EM1 CGTase a single F196G mutation would not cause any conformational changes that might affect the pH optima of the different reactions. In contrast, we cannot exclude that deletion of 127 amino acids from the C-terminus of CGTase could change the pH optimum. For instance, deletion of 10 and 13 amino acids from the C-terminus of a *Bacillus* sp. 1011 CGTase reduced the pH optimum for starch degradation from pH 5–11 for the wild-type CGTase to pH 5–9 and 5–7, respectively, for the truncated proteins (Kimura et al., 1989). Thus, the protonation state of the catalytic residue Glu258, which determines the pH optima for cyclization and hydrolysis (Wind et al., 1997), might be influenced by the rearrangements resulting from the 127-amino-acid deletion.

Maximum CGTase wild-type cyclization activity was observed at 80–85°C, whereas maximum CGTase F196G(Δ'DE) activity was observed at 50–55°C (Fig. 5). The high temperature optimum of the wild-type CGTase from *T. thermosulfuri-*

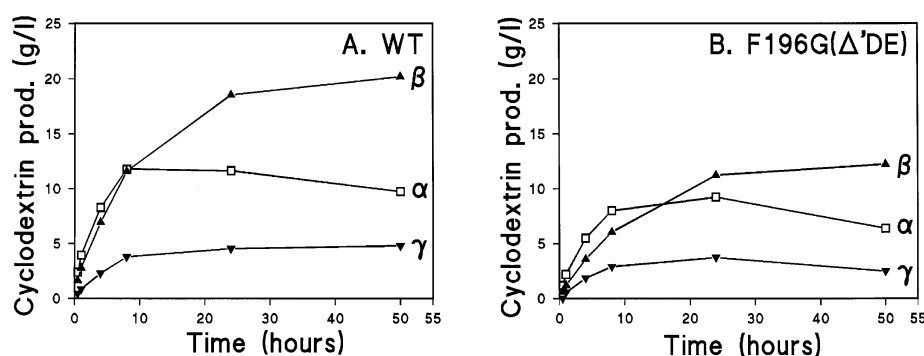


**Table 2. Specific enzyme activities and pH optima for *T. thermosulfurigenes* EM1 wild-type CGTase and mutant F196G( $\Delta'$ DE).** Cyclization activity is shown as  $\beta$ -cyclodextrin-forming activity.

CGTase	Specific enzyme activities						
	cyclization	coupling	disproportionation	saccharifying		pH optima	
				at pH 6.0	at pH optimum	cyclization	saccharifying
	U/mg						
Wild-type	165	45	330	25	30	4.5–6.5	4.0–5.0
F196G( <i>Δ'</i> DE)	40	3	95	55	65	5.5–6.5	5.0–5.5

**Table 3. Starch conversion by *T. thermosulfurigenes* EM1 wild-type CGTase and mutant F196G( $\Delta'$ DE).** Proteins (0.1 U/ml  $\beta$ -cyclodextrin forming activity) were incubated for 45 hours with 10% Paselli WA4. Starch conversion into cyclodextrins or linear sugars (glucose, maltose and maltotriose) are shown relative to the initial amount of starch.

CGTase	Conversion of starch into cyclodextrins	Product ratio			Conversion of starch into linear sugars
		$\alpha$	$\beta$	$\gamma$	
	%				
Wild-type	35	28	58	14	11
F196G( $\Delta'$ DE)	21	30	58	12	18

**Fig. 4. Cyclodextrins formed during incubation of the wild-type CGTase from *T. thermosulfurigenes* EM1 (A) and mutant F196G( $\Delta'$ DE) (B).** Proteins (0.1 U/ml  $\beta$ -cyclodextrin forming activity) with 10% (mass/vol.) Paselli WA4 starch for 45 hours at pH 6.0 and 60°C.  $\square$   $\alpha$ -cyclodextrin,  $\blacktriangle$   $\beta$ -cyclodextrin,  $\blacktriangledown$   $\gamma$ -cyclodextrin.

genes EM1, when compared to mesophilic CGTases, has been attributed to a combination of factors involving novel hydrogen bonds, apolar contacts, salt-bridges and Gly to Ala/Pro substitutions (Knegt et al., 1996). Most of the amino acids involved in these novel interactions were present in mutant F196G( $\Delta'$ DE). The decrease in temperature optimum of the mutant enzyme, therefore, must be caused by the truncation of domain E and part of domain D, which exposes hydrophobic residues to the solvent which is thermodynamically unfavorable.

CGTases consist of five domains (A–E), whereas  $\alpha$ -amylases possess only the first three domains (A–C). The A and B domains contain the  $(\beta/\alpha)_8$ -barrel, the active-site cleft, and the substrate-binding residues (Svensson et al., 1994). No specific function has been assigned to domain C, but mutations in  $\alpha$ -amylase from *B. stearothermophilus* indicate that it is required for starch hydrolysis activity (Holm et al., 1990). Analysis of a series of mutants of a *B. stearothermophilus*  $\alpha$ -amylase showed that C-terminal truncations of increasing length progressively reduced the specific activity for starch hydrolysis (Vihinen et al., 1994). It has been proposed that in  $\alpha$ -amylases domain C plays

an important role in starch hydrolysis, by orientating the active-site cleft of domain A correctly with respect to the amylose chain. The function of the D domain of CGTase is not known. Domain E is involved in raw starch binding by CGTase (Svensson et al., 1989; Penninga et al., 1996; Svensson, 1994). Domain E of the *B. circulans* 251 CGTase contains two MBS; MBS1 is involved in raw starch binding and MBS2 in guiding the starch chain into the active site. MBS2 also plays a role in cyclodextrin product inhibition (Penninga et al., 1996). This explains the severely reduced raw-starch-binding capacity of mutant F196G( $\Delta'$ DE). The wild-type CGTase from *T. thermosulfurigenes* EM1 displayed similar raw-starch-binding properties ( $B_{\max} = 1$ ,  $K_{50} = 0.7\%$ ) as the CGTase from *B. circulans* strain 251 ( $B_{\max} = 1$ ,  $K_{50} = 0.8\%$ ; Penninga et al., 1996), whereas mutant F196G( $\Delta'$ DE) did not bind any raw starch at all.  $B_{\max}$  is the maximal fraction of the protein bound to raw starch and the  $K_{50}$  is the percentage of raw starch at which half of the enzyme is bound (Penninga et al., 1996).

Conflicting reports have appeared about the role of the C-terminus in CGTase cyclization activity. Removal of the

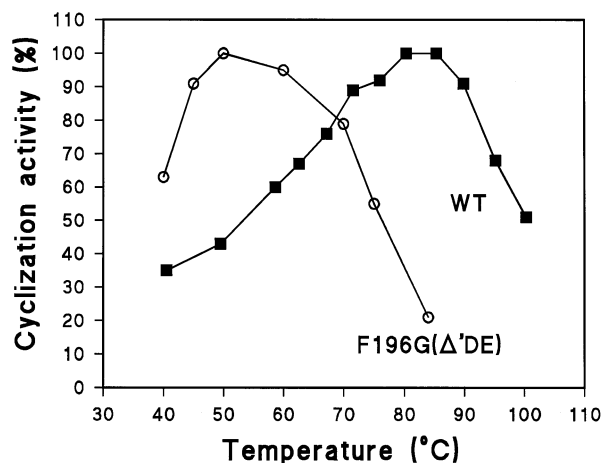


Fig. 5. Effect of temperature on cyclization activity of the wild-type CGTase from *T. thermosulfurigenes* EM1 and mutant F196G( $\Delta'$ DE).

C-terminal 90 amino acids of the *K. pneumoniae* CGTase had little effect on activity or product specificity (Bender, 1990a), whereas deletion of a mere 10–13 residues of the *Bacillus* sp. 1011 CGTase already caused significant changes in product specificity (Kimura et al., 1989). The present study clearly shows that part of the D domain and the complete E domain are dispensable in the *T. thermosulfurigenes* EM1 CGTase. However, the C-terminus was indispensable for activity in the  $\beta$ -CGTases from *Bacillus* sp. 1011 (Kimura et al., 1989), *B. circulans* var. *alkalophilus* (Hellman et al., 1990) and *B. circulans* strain 251 (Penninga, D., unpublished results).

## CONCLUSIONS

Mutant F196G of the highly thermostable *T. thermosulfurigenes* EM1 CGTase was constructed using site-directed mutagenesis. Due to a frameshift mutation, the E domain and part of the D domain had also been deleted from this protein. With respect to the domain structure and identity of the residue at position 196, this mutant CGTase is more similar to  $\alpha$ -amylases; however, it contains an additional C-terminal structure compared to  $\alpha$ -amylases. The C-terminal deletion yielded a protein unable to bind to raw starch and displaying a strongly reduced thermostability. Concomitantly, the cyclization, coupling and disproportionation activities became severely reduced, whereas a doubling of the saccharifying activity was observed. This resulted in a decreased conversion of starch into cyclodextrins and increased conversion into linear oligosaccharides. These biochemical characteristics indicate a shift from CGTase to  $\alpha$ -amylase specificity. Nevertheless, the mutant still produces cyclodextrins. The data provide a firm basis for analysis of other factors determining CGTase and  $\alpha$ -amylase product specificity in future work.

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